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Use of metal chelate affinity chromatography and membrane-based ion-exchange as clean-up procedure for trace residue analysis of tetracyclines in animal tissues and egg

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Abstract

A new and efficient procedure for the clean-up of tetracycline residues in animal tissues and egg prior to reversed-phase high-performance liquid chromatography is described. The principal steps involve homogenization of the tissues in sodium succinate buffer and methanol, followed by centrifugation and clean-up with metal chelate affinity chromatography (MCAC). After further concentration on an Empore extraction membrane with cation-exchange properties, the sample is analysed by HPLC with fluorescence detection. The method was tested on porcine kidney and muscle, bovine liver and whole chicken's egg. The recoveries were determined from spiked tissues for oxytetracycline, tetracycline, chlortetracycline and doxycycline and ranged from 40 to 70%, with repeatabilities below 10% R.S.D.. The analytical responses were linear in the range up to at least 1000 ng/g. The detection limits of the method were estimated at 0.42 ng/g of oxytetracycline, 0.49 ng/g of tetracycline, 0.66 ng/g of chlortetracycline and 1.38 ng/g of doxycycline in porcine muscle, using signal-to-noise ratios of 4:1. Similar detection limits were estimated for kidney, liver and egg. The measured limits of quantification were 2 ng/g for oxytetracycline, 3 ng/g for tetracycline, 4 ng/g for chlortetracycline and 5 ng/g for doxycycline in porcine kidney. The advantage of this method over existing methods is its increased limit of detection.

Keywords: Oxytetracycline; Tetracycline; Chlortetracycline; Doxycycline

1. Introduction

Tetracycline antibiotics (TCs), such as oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC) are commonly administered to food-producing animals as veterinary drugs because of their broad spectrum activity and cost effectiveness. Their usage may result in residues in food products of animal origin, often due to the improper observance of withdrawal times. For this reason, the EU has laid down maximum residue limits (MRLs) for OTC, TC and CTC which have been set at 100 ng/g in muscle, 200 ng/g in egg, 300 ng/g in liver and at 600 ng/g in kidney [1]. The MRL for DC has been temporary set at the same limit and is still under consideration.

Extraction and clean-up techniques for TCs analysis in foods of animal origin were recently reviewed by Oka et al. [2]. A common clean-up technique is solid-phase extraction (SPE) with C_{18} cartridges. However, because these cartridges often present

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problems of tailing and poor recoveries, a pretreatment of the C_{18} cartridges with EDTA [3] or silylation reagent [4] was shown to be necessary. To overcome these problems, metal chelate affinity chromatography (MCAC) has been developed [5], based on the complex formation of cations with TCs. However, this method needs further concentration of the MCAC eluate to enable low detection limits. Methods which can achieve low detection limits can be very useful, e.g., to establish blank values for candidate reference materials. Moreover, the MCAC eluates develop a precipitate that can clog and significantly shorten the lifetime of a HPLC column [7]. Degroodt et al. [6] use C_{18} SPE to further concentrate the MCAC eluate, while Carson [7] describes the use of ultrafiltration as further deproteinization step, but both procedures seem to be time-consuming. The purpose of the present study was to develop a rapid and appropriate method for the concentration of TCs residues in the MCAC eluate. Since TCs have amphoteric characteristics [8], cation-exchange methods can be considered. The use of solid-phase bonded silica with cation-exchange properties packed in cartridges has already been reported for the extraction and clean-up of TCs in honey, where a tandem cartridge system of C_{18} and COOH cartridges is described [9]. We investigated the potential of Empore cation-exchange extraction membranes for concentration of TCs in the MCAC eluate. These extraction membranes contain small sorbent particles embedded in an inert matrix of polytetrafluoroethylene fibrils. They are available in the same diameters as HPLC solvent filters and were first designed for the environmental analysis of pollutants in water [10]. They have also been applied in the field of therapeutic drug monitoring for sample preparation from serum [11] and urine [12]. Recently, the membranes were applied for the extraction of illegally used growth promoters from cattle urine, such as anabolic steroids [13] and β-agonists [14]. To our knowledge, no application of these membranes has been described yet for TCs residue analysis. The most important advantages of the extraction membranes over the conventional extraction cartridges are the rapid processing and the small elution volume required, resulting in highly concentrated eluates.

2. Experimental

2.1. Apparatus and chromatographic conditions

The HPLC system consisted of a Waters Model 600-MS pump (Waters Chromatography, Milford, MA, USA), a Waters Model U6K injector and a Waters Model 470 fluorescence spectrophotometer. Fluorescence was measured after post-column addition of 5% (m/v) zirconyl chloride octahydrate in HPLC grade water [15]. The post-column reagent was kept in an amber bottle at 4°C for stability reasons. The excitation wavelength was set at 406 nm while emission was measured at 515 nm. The post-column configuration consisted of a Waters Model 610 pump, a mixing tee U-466 (Upchurch Scientific, Oak Harbor, WA, USA) and a Waters reactor coil of 600 μl. A 40 p.s.i. back-pressure regulator P-748 (Upchurch Scientific) was placed after the flow-cell to obtain a stable baseline. Detector signals were processed with a HP 3396 Series II Integrator (Hewlett-Packard, Waldbronn, Germany). A RP polymeric column type PLRP-S, 100 Å, 8 μm, 250×4.6 mm I.D. was used in combination with a PLRP-S guard cartridge of 5×3.0 mm (Polymer Laboratories, Church Stretton, UK). The mobile phase was prepared with HPLC grade solvents (BDH, Poole, UK) and contained 0.01 *M* oxalic acid in water, adjusted to pH 2.0 with 4 *M* HCl (A), and acetonitrile (B). A gradient solvent programme (A–B, 85:15 to 60:40, v/v, in 16 min) was run. The flow-rate was set at 1 ml/min and was identical for both HPLC pumps. All TCs-containing solutions were protected from light.

2.2. Reagents

All chemicals were of analytical grade and were from Janssen Chimica (Beerse, Belgium), Merck (Darmstadt, Germany) or UCB (Drogenbos, Belgium). Deionized distilled water was used throughout, unless stated otherwise. OTC and hydrochloride salts of TC and CTC were secondary standards, purchased from Janssen Chimica. Demeclocycline hydrochloride (DMCTC), doxycycline hydrochloride (DC) and zirconyl chloride octahydrate were obtained from Fluka Chemie (Buchs, Switzerland).

Chelating Sepharose Fast-Flow suspended in 20% ethanol, as purchased from Pharmacia Biotech (Uppsala, Sweden), was used for affinity chromatography. The sodium succinate buffer contained 0.1 *M* succinic acid and was adjusted to pH 4.0 using 10 *M* NaOH. The McIlvaine buffer was prepared by dissolving 12.9 g citric acid monohydrate and 10.9 g Na_2HPO_4 in 1 l water. The McIlvaine–EDTA–NaCl buffer consisted of 0.1 *M* EDTA and 0.5 *M* NaCl in McIlvaine buffer. Empore SDB–RPS [poly(styrene–divinylbenzene)-RP sulfonated] and SCX (strong cation exchanger) were from the 3M Company (St. Paul, MN, USA), marketed by Varian (Harbor City, CA, USA). Both membranes contain 100% polymeric material modified with sulfonic acid groups, displaying RP and cation interactions, but SDB–RPS has much lower sulfonic acid functionality than the strong cation exchanger. The 47-mm diameter membrane was cut into 13-mm disks using a cork borer and placed in a FM 013/0 filter holder made of stainless steel (Schleicher and Schuell, Dassel, Germany), as described by our group [14]. A 10-ml disposable syringe (Becton Dickinson, NJ, USA) was placed on top of the filter holder and the syringe plunger was used to put the MCAC eluate through the membrane. Stock standard solutions of TC, OTC, CTC, DC and DMCTC (1 mg/ml) were prepared in HPLC grade methanol and stored at −20°C. The working standard solutions combining OTC, TC, CTC and DC were prepared in HPLC grade water immediately before use to fortify the tissues. The working I.S. solution (DMCTC 25 μg/ml) was prepared in HPLC grade water and stored at 4°C.

2.3. Procedure

Three grams of minced porcine kidney, porcine muscle, beef liver tissue or whole chicken's egg were placed in a 50-ml screw-capped amber centrifuge tube. For recovery studies, the tissues were spiked at this point with the working standard solutions. A 20-ml volume of 0.1 *M* sodium succinate buffer (pH 4.0) was added and the tube was vortexed for 1 min. The contents were then shaken for 10 min on a horizontal shaker. Next, 20 ml of methanol were added as a deproteinization solution. The tube was placed in an ultrasonic bath for 5 min and centrifuged for 10 min at 2666 *g* at 4°C. The supernatant was filtered through a Whatman 541 filter paper. The clear supernatant was applied directly onto a previously prepared MCAC column, as described elsewhere [16]. The column was washed sequentially with 2 ml of 0.1 *M* sodium succinate buffer, 2 ml of water, 2 ml of methanol and 2 ml of water. Finally, 0.5 ml of McIlvaine–EDTA–NaCl buffer was applied to the column and the TCs were eluted with 3.0 ml of McIlvaine–EDTA–NaCl buffer. The pH of the MCAC eluate was adjusted to pH 1.3 by adding 400 μl of 4 *M* HCl. This eluate was applied directly onto the SDB–RPS extraction membrane which had first been preconditioned by pushing successively 2 ml of methanol and 2 ml of 0.1 *M* HCl through the membrane with the use of the syringe. During this step, air must not reach the membrane. It is important to apply the pH-adjusted eluate directly onto the membrane in order to prevent crystallization of EDTA. After application of the MCAC eluate, the membrane was washed with 1 ml of 0.1 *M* HCl and the TCs were eluted with 4×250 μl of HPLC grade methanol containing concentrated (25%) ammonia (97:3, v/v). The extract was evaporated to dryness under nitrogen (40°C). The dried sample was reconstituted with 250 μl of 0.01 *M* oxalic acid in HPLC grade water, vortexed and ultrasonicated. A 100-μl aliquot was injected into the HPLC system. For the study of the recovery efficiency, 20 μl of 25 μg/ml DMCTC was added at this stage as an external standard: the peak-area ratio of the TCs/DMCTC was compared with the corresponding ratio obtained from a calibration standard solution.

3. Results and discussion

3.1. MCAC procedure

The initial MCAC procedure used was described by Carson [7] for the clean-up of TCs in milk. Briefly, the milk sample was homogenized with 0.1 *M* sodium succinate buffer (pH 4.0), centrifuged and the clear supernatant was applied to a MCAC column. When applying this procedure to tissues, a further optimization was necessary. This included

Table 1
Influence of different extraction parameters on the recovery (%) from the MCAC column of four TCs in porcine kidney (spiked at 600 ng/g), using 20 ml of extraction buffer and 20 ml of deproteinization solution

Parameter	OTC	TC	CTC	DC
Extraction buffer				
McIlvaine, pH 4.0	41	10	36	48
Citric acid 0.1 *M*–Na_2HPO_4 0.2 *M* (62:38, v/v), pH 4.0	45	10	37	47
Sodium succinate 0.1 *M*, pH 4.0	71	60	70	72
Deproteinization solution				
Acetonitrile	ND[a]	ND	ND	ND
Methanol	72	57	69	73

[a] Not detected.

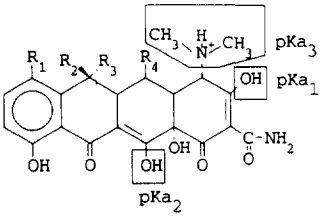
studies of the influence of the extraction buffer and of the deproteinization solution on the recovery. For both experiments, 3 g of tissue was used. Some experiments were carried out to study the influence of the amount of sample on the recovery. These results showed a decreasing trend in recovery with increasing sample amount (3, 4 and 5 g). Therefore, 3 g of sample was chosen for the final procedure. In all experiments the MCAC eluate was injected directly into the HPLC system. No interfering endogenous peaks in the chromatogram were observed. The results for kidney tissue are illustrated in Table 1. Optimal recoveries were obtained using 20 ml of 0.1 *M* sodium succinate extraction buffer and 20 ml of methanol as deproteinization solution. Additional experiments showed that an ultrasonication step of 5 min after addition of the deproteinization solution was critical for good recoveries. Consequently, this method was utilized in the further study of the membrane-based ion-exchange procedure. The MCAC method performance data (recovery and linearity) for kidney tissue are listed in Table 2. The recoveries were obtained from spiked kidney at the actual MRL of 600 ng/g and the repeatability was expressed as the relative standard deviation (R.S.D.) ($n=7$). The linearity of the assay (correlation coefficient r^2) was checked by analysing kidney spiked with 200, 400, 600, 800 and 1000 ng/g ($n=2$). The detection limits (LODs) in kidney were estimated at 20 ng/g, 27 ng/g, 28 ng/g and 57 ng/g for OTC, TC, CTC and DC, respectively, using *S/N* of 4/1.

Table 2
MCAC performance data for porcine kidney spiked at 600 ng/g

	Recovery (Mean±R.S.D., $n=7$) (%)	Linearity (r^2) ($n=2$)
OTC	69±5.6	0.9960
TC	59±5.9	0.9986
CTC	68±6.5	0.9973
DC	76±5.1	0.9977

3.2. Membrane-based ion-exchange procedure

The detection limits of the MCAC procedure were not low enough for our purpose, as the 3.0 ml of McIlvaine–EDTA–NaCl buffer used for elution of the MCAC column was difficult to evaporate. Our experience with concentrating the MCAC eluate using conventional techniques was unfavorable. Analytical recovery was low and inconsistent on a commonly used silica-based C_{18} cartridge, probably because of TCs property to bind with residual silanol groups in the stationary phase. The Empore membranes are available either with silica-based or polymeric particles. The latter were very interesting for our purpose. Opposed to silica-based membranes they do not contain residual silanol groups and they are stable at pH extremes (pH 0–14). In a first experiment, two different cation-exchange sorbents, SDB–RPS and SCX, were examined. These cation-exchange membranes were expected to be effective because TCs contain a dimethylaminogroup. The pK_a values of TCs in aqueous solution are approximately pK_{a_1} 3.3, pK_{a_2} 7.5 and pK_{a_3} 9.3 (Fig. 1) [8]. Since the pH of the McIlvaine–EDTA–NaCl buffer was 3.7, an adjustment of the pH to 1.3 using 4 *M* HCl was necessary in order to protonate the TCs. For all experiments, 3% ammonia in methanol (v/v) was used as elution solvent. No other basic eluents such as NaOH or KOH in methanol were tested, since ammonia has a low boiling point (−33°C). This was important because the stability of TCs is poor under strong alkaline conditions, especially upon heating. Secondly, the suitability of methanol, ethanol, isopropanol and ethyl acetate, containing 3% ammonia, was investigated as elution solvent from the SDB–RPS membrane. The results for kidney spiked at 600 ng/g are summarized in Table 3. The recovery on



Compound	R_1	R_2	R_3	R_4
TC	H	OH	CH_3	H
OTC	H	OH	CH_3	OH
CTC	Cl	OH	CH_3	H
DMCTC	Cl	OH	H	H
DC	H	H	CH_3	OH

Fig. 1. Structural formulas of the tetracycline antibiotics studied: tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DC) and demeclocycline (DMCTC).

SCX was lower compared to SDB–RPS, probably because of the strong binding of the quaternary ammonium group on the SCX. The bond is probably so strong that the TCs do not elute. The effect of the elution volume on the recovery of TCs in spiked kidney from the SDB–RPS membrane is demonstrated in Fig. 2. This figure shows that four elution steps of 250 μl are necessary to obtain quantitative recovery for OTC, and three elution steps of 250 μl for TC, CTC and DC. Other experiments were carried out on the influence of the concentration of the wash solution. A 0.1 *M* HCl solution (pH 1) and a 0.01 *M* HCl solution (pH 2) were compared. No difference in recovery was observed. The application of an extra methanol wash step (200 μl) after the

Table 3
Influence of cation-exchange sorbent and elution solvent on the recovery (%) of porcine kidney spiked at 600 ng/g

Parameter	OTC	TC	CTC	DC
Sorbent				
SCX	41	34	34	30
SDB-RPS	65	54	59	63
Elution solvent				
Ethyl acetate	6	4	ND[a]	ND
Isopropanol	38	31	33	50
Ethanol	63	52	51	62
Methanol	65	56	51	69

[a] Not detected.

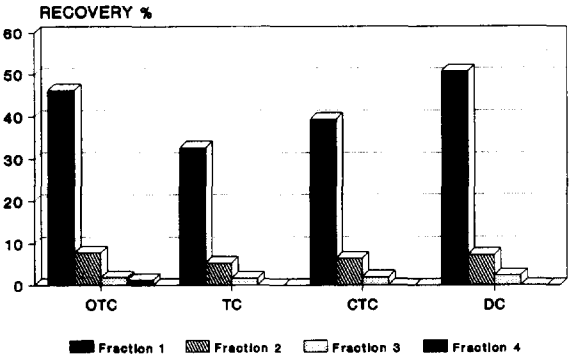


Fig. 2. Recovery of TCs in porcine kidney (spiked at 600 ng/g) from the SDB–RPS extraction membrane using different 250-μl elution fractions of methanol–ammonia (97:3, v/v).

HCl wash was also studied. Minimal losses of TCs ($\leq$1.5%), depending on the kind of TCs, were detected. This methanol wash step did not result in an extra clean chromatogram.

A representative chromatogram of a blank kidney sample and a sample spiked at 50 ng/g OTC, TC, CTC and DC is shown in Fig. 3. A gradient programme was necessary to obtain good peak symmetry. The final extraction recoveries for the different tissues obtained at the actual MRL are shown in Table 4. These values are the results of repetitive analyses, obtained on several days, and the precision (between-day precision) is expressed as the R.S.D. (%). The linearity of the assay was checked using spiked tissue with spike levels including the MRL. The concentrations examined were 0, 200, 400, 600 and 800 ng/g and 0, 50, 100, 150 and 200 ng/g for kidney and muscle, respectively. The concentration levels examined for both liver and egg were 0, 100, 200, 300 and 400 ng/g. The correlation coefficients r^2 of the five points calibration curves in matrix and the estimated LODs (*S/N* of 4/1) are given in Table 5. The limit of quantification (LOQ) was defined as the lowest concentration in kidney of OTC, TC, CTC and DC for which the method is validated with an accuracy and precision that fall within the ranges recommended by the EC [17,18] for the analysis of a reference material. The LOQ was determined by analysing spiked kidney samples ($n=6$) at a level of 2 ng/g OTC, 3 ng/g TC, 4 ng/g CTC and 5 ng/g DC. The range for the accuracy for an analyte content of >1 ng/g to 10 ng/g is −30%

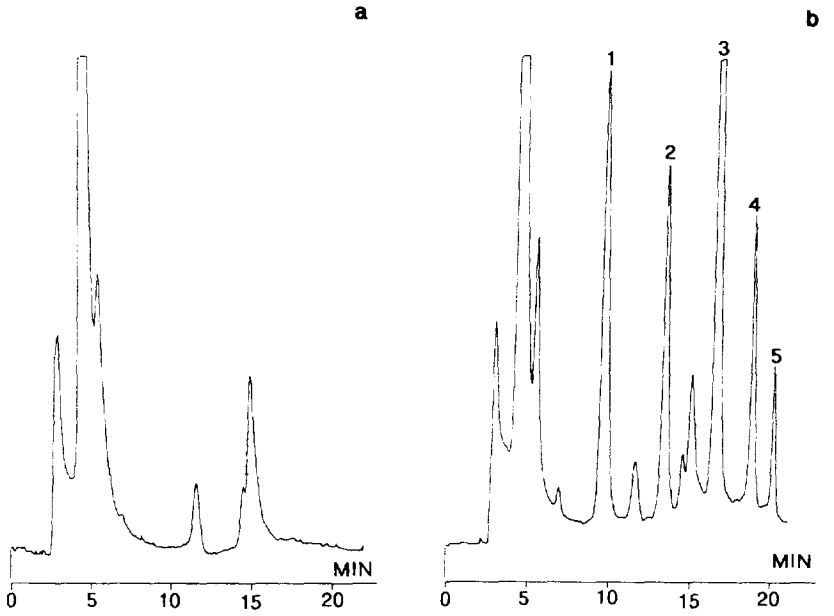


Fig. 3. Chromatogram of (a), blank porcine kidney and (b), porcine kidney spiked at 50 ng/g of OTC, TC, CTC, DC and 20 μl of 25 μg/ml DMCTC (I.S.). Peaks: 1 = OTC; 2 = TC; 3 = DMCTC; 4 = CTC and 5 = DC; processed according to the MCAC and SDB–RPS extraction membrane procedure (Section 2.3). Conditions: column, PLRP-S 8 μm (250×4.6 mm I.D.) with a 5×3.0 mm PLRP-S guard cartridge; mobile phase, 0.01 *M* oxalic acid (pH 2.0)–acetonitrile gradient; flow-rate, 1 ml/min; fluorescence detection, λ_{ex} = 406 nm, λ_{em} = 515 nm.

Table 4
Extraction recoveries for different tissues spiked at the MRL using the MCAC and SDB–RPS extraction membrane clean-up procedure

	Pork kidney		Pork muscle		Beef liver		Chicken's egg	
	n	Mean±R.S.D. (%)	*n*	Mean±R.S.D. (%)	*n*	Mean±R.S.D. (%)	*n*	Mean±R.S.D. (%)
OTC	10	59±2.7	6	69±5.2	6	58±4.8	6	50±4.2
TC	10	45±4.5	6	54±7.3	6	39±6.9	6	45±1.8
CTC	10	46±10.8	6	53±4.9	6	42±6.9	6	37±3.0
DC	10	58±7.9	6	66±6.0	6	53±9.3	6	53±2.9

Table 5
Regression analysis of matrix calibration curves (5 points) and LODs (ng/g) for different tissues using the MCAC and SDB–RPS extraction membrane clean-up procedure

	Pork kidney		Pork muscle		Beef liver		Chicken's egg	
	r^2	LOD	r^2	LOD	r^2	LOD	r^2	LOD
OTC	0.9911	0.61	0.9990	0.42	0.9956	0.68	0.9990	0.21
TC	0.9805	1.20	0.9976	0.49	0.9912	1.01	0.9981	0.23
CTC	0.9915	0.92	0.9949	0.66	0.9923	1.05	0.9985	0.39
DC	0.9966	3.00	0.9898	1.38	0.9919	2.35	0.9961	0.80

Table 6
Limit of quantification of OTC, TC, CTC and DC in porcine kidney ($n=6$)

	Theoretical concentration (ng/g)	Measured concentration (mean±S.D.) (ng/g)	R.S.D. (%)	$R.S.D._{max}$ (%)
OTC	2	2.16±0.14	6.6	27.2
TC	3	2.91±0.69	23.8	25.6
CTC	4	3.26±0.61	18.7	24.5
DC	5	3.55±0.81	22.7	23.7

to +10%. The mean concentration fell within these ranges (Table 6). The precision ($R.S.D._{max}$, %) for analyses carried out under repeatability conditions are two-thirds of the values calculated according to the Horwitz equation [17,18]. Table 6 shows that the obtained R.S.D. (%) values are below these $R.S.D._{max}$ values.

Table 5 demonstrates that the Empore cation-exchange clean-up is a very effective procedure to reach increased LODs. The study further demonstrates that the method is applicable to a wide range of tissues, including muscle, liver, kidney and egg, using the same sample preparation method.

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